



Short communication

Measurement of monoterpenes and sesquiterpenes in serum, plasma, and rumen fluid from sheep[☆]R.E. Estell^{a,*}, S.A. Utsumi^{b,1}, A.F. Cibils^b^a USDA, ARS, Jornada Experimental Range, Las Cruces, NM, USA^b Animal and Range Science Department, New Mexico State University, Las Cruces, NM, USA

ARTICLE INFO

Article history:

Received 25 September 2009

Received in revised form 11 February 2010

Accepted 12 March 2010

Keywords:

Plasma

Recovery

Rumen fluid

Serum

Shrub

Terpene

ABSTRACT

Studies involving the consumption, metabolism, and elimination of terpenes by small ruminants consuming terpene-laden shrubs as well as those exploring the potential for natural products as rumen modifiers could benefit from a procedure that measures terpenes in both blood and rumen fluid and that is suitable for either serum or plasma. The objective of this study was to modify an existing procedure for plasma utilizing solid phase extraction/gas chromatography, and extend its use for measurement of structurally diverse mono- and sesquiterpenes in three fluids (serum, plasma, and rumen fluid) from sheep. Generally, terpene recovery was lower from rumen fluid than from serum or plasma, although the extent and direction of differences varied among chemicals. Fourteen terpenes (camphene, β -pinene, α -terpinene, *p*-cymene, *cis*- β -ocimene, 1,8-cineole, γ -terpinene, terpinolene, linalool, camphor, longifolene, β -caryophyllene, α -humulene, and caryophyllene oxide) were recovered from serum at near unity. Recovery from rumen fluid was lower than that for serum or plasma for most terpenes, but eight (*p*-cymene, 1,8-cineole, *cis*-sabinene hydrate, terpinolene, borneol, terpin-4-ol, α -terpineol, and caryophyllene oxide) were recovered at near unity. Yet, 15 terpene recoveries were below 0.75 ng/ng (tricyclene, α -pinene, camphene, sabinene, β -pinene, myrcene, 2-carene, 3-carene, α -terpinene, *cis*- β -ocimene, limonene, γ -terpinene, longifolene, β -caryophyllene, and α -humulene). Oxygenated monoterpenes were typically recovered in greater quantities and hydrocarbon monoterpenes were least effectively recovered with this method. This procedure allows for simultaneous measurement and recovery adjustment of a number of terpenes from serum, plasma, and rumen fluid of sheep.

Published by Elsevier B.V.

1. Introduction

Shrubs encroaching into grasslands reduce forage availability for livestock, increase erosion, and reduce water potential. Many invasive species contain high levels of terpenes, yet little is known about the mechanisms by which ruminants cope with these secondary compounds after consumption. Assessing the fate of terpenes ruminally (*i.e.*, microbial effects) and

Abbreviations: GC, gas chromatograph; MS, mass spectrometer; RT, retention time; SPE, solid phase extraction; SPME, solid phase microextraction.

[☆] Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

* Corresponding author. Tel.: +1 575 646 4842; fax: +1 575 646 5889.

E-mail address: restell@nmsu.edu (R.E. Estell).

¹ Present address: Department of Animal Science, Michigan State University, W.K. Kellogg Biological Station, Hickory Corners, MI, USA.

metabolically (absorption, metabolism, and elimination) is crucial for pharmacokinetic studies. Furthermore, interest in natural products as rumen modifiers (e.g., plant extracts containing essential oils) has escalated in recent years, particularly in countries that have banned traditional modifiers. A number of studies (e.g., Newbold et al., 2004; Cardoza et al., 2004) have examined effects of various essential oil components and extracts on intake, digestion, microbial populations, and fermentation. This topic was the subject of a recent special issue of Animal Feed Science and Technology (vol. 145, 2008). Once beneficial compounds/blends are identified, their residence time and disappearance from the rumen and movement into the bloodstream must be determined. Although a variety of techniques exist for measuring terpenes in plant materials, few methods are available for quantifying terpenes in blood and rumen fluid of livestock. The SPE procedure of Kimball et al. (2004) is an accepted protocol for plasma terpenes, but has not been tested on serum and was developed using three monoterpenes in sagebrush (*Artemisia tridentata*). Rumen terpenes have been examined using solvent extraction (Chizolla et al., 2004; Broudiscou et al., 2007) and SPME (Malecky and Broudiscou, 2009). It would be useful to have a compatible method for serum, plasma, and rumen fluid. The objective of this study was to determine the suitability of a procedure (modification of Kimball et al., 2004) for use with all three fluids and a broad range of structurally diverse mono- and sesquiterpenes varying in molecular weight, ring number, degree of saturation, and oxygenation.

2. Materials and methods

2.1. Sample collection, processing, and storage

Animal handling procedures and experimental protocols were approved by the New Mexico State University Institutional Animal Care and Use Committee. Six mature Rambouillet ewes (not pregnant or lactating) were used for blood and rumen fluid sampling. Ewes were part of a group housed in 5 m × 20 m pens with a shaded roof and free access to fresh water and trace mineral salt blocks. Animals were fed a basal diet of alfalfa hay each morning at 07:00 h (typically consumed within 3–4 h). Rumen fluid and blood for serum and plasma separation were collected between 08:00 and 10:00 h. Approximately 100 ml of blood were collected from each ewe (50 ml each for plasma and serum) by jugular venipuncture into 10 ml vacuum tubes (Corvac™ serum separator and Monoject™ 0.15 g/ml EDTA, Kendall, Ontario, CA, USA). Blood was transported to the laboratory within 25 min. Plasma tubes were processed immediately and serum tubes were allowed to coagulate for 30 min. All blood samples were centrifuged (Sorvall RT6000, Thermo Electron Corp., Ashville, NC, USA) at 1500 × g for 20 min at 10 °C. Serum and plasma were immediately decanted using transfer pipettes into 20 ml plastic vials and stored at –20 °C. Rumen fluid (100 ml per animal) was collected via oral lavage with a stomach tube and vacuum pump and centrifuged at 27,000 × g for 20 min at 10 °C. Rumen fluid was then decanted into 20 ml plastic vials (to avoid repeated thawing/freezing throughout study) and stored at –80 °C. Samples were thawed for 24 h at 4 °C prior to analysis, and rumen fluid was re-centrifuged at 2000 × g for 15 min at 4 °C prior to analysis.

2.2. Test compounds

We selected 25 mono- and sesquiterpenes representing a wide range of saturation, oxygenation, and ring numbers for testing recovery. These compounds are also commercially available and are key components of shrub species (e.g., *A. tridentata*, *Flourensia cernua*, several *Juniperus* spp.) currently being examined at various locations with respect to shrub intake and biocontrol by small ruminants. A monoterpene and a sesquiterpene (2-carene and longifolene) were also included because they have been used as internal standards for a number of studies at this location. Tricyclene (99/100 g), α -pinene (98/100 ml), camphene (95/100 g), β -pinene (99/100 ml), myrcene (90/100 ml), 2-carene (97/100 ml), 3-carene (95/100 ml), α -terpinene (95/100 ml), *p*-cymene (99/100 ml), limonene (97/100 ml), γ -terpinene (98/100 ml), linalool (97/100 ml), camphor (96/100 g), borneol (99/100 g), caryophyllene oxide (99/100 g), α -terpineol (90/100 ml), longifolene (99/100 ml), and β -caryophyllene (90/100 ml) were purchased from Sigma–Aldrich (St. Louis, MO, USA). 1,8-cineole (98/100 ml), *cis*- β -ocimene (70/100 ml), *cis*-sabinene hydrate (98/100 g), terpinolene (90/100 ml), and α -humulene (98/100 ml) were purchased from Fluka (Milwaukee, WI, USA). Sabinene (90/100 ml) and terpin-4-ol (97/100 ml) were purchased from Indofine Chemical Co. (Hillsborough, NJ, USA) and Acros Organics (Morris Plains, NJ, USA), respectively.

A stock solution of each compound was prepared in pure ethanol (Aaper Alcohol and Chemical Co., Shelbyville, KY, USA) at 10,000 ng/ μ l (adjusted for purity and stored in 7 ml amber vials at –20 °C) and diluted to ~50 ng/ μ l (working solution). These individual working solutions were subjected to GC/MS as described below, and the RT of each compound and any impurity peaks were determined. The 25 terpenes were then separated into one of four groups based on maximizing distance among individual peaks and minimizing interferences from impurity peaks in the commercially purchased compounds. The purpose of creating groups was to examine a wide assortment of terpenes commonly encountered by browsing ruminants while maintaining a manageable number of samples. Each group consisted of six to nine compounds with good separation among compounds and minimal impurity peaks (considering both RT and peak size) that could potentially interfere with recovery estimates of other compounds in subsequent tests. Also, 2-carene and longifolene (a mono- and a sesquiterpene) were added to each group to examine method consistency across runs. Groups consisted of α -pinene, camphene, β -pinene, 2-carene, *p*-cymene, terpinolene, borneol, longifolene, and β -caryophyllene (Group 1) sabinene, myrcene, 2-carene, limonene, camphor, longifolene, and α -humulene (Group 2) tricyclene, 2-carene, 3-carene, 1,8-cineole, γ -terpinene, linalool, terpin-4-ol, longifolene, and caryophyllene oxide (Group 3), and 2-carene, α -terpinene, *cis*- β -ocimene, *cis*-sabinene hydrate, α -

terpineol, and longifolene (Group 4). These mixtures were then prepared in ethanol (stock solutions; 5000 ng/ μ l) and diluted to 100 ng/ μ l working solutions for all subsequent tests.

2.3. Extraction procedure

The SPE column used in this study was Extract-CleanTM C₁₈-HC, 500 mg sorbent/8 ml reservoir (Grace Davison Discovery Sciences, Deerfield, IL, USA). Columns were placed on a 12-port vacuum manifold and conditioned as described by Kimball et al. (2004) with 5 ml of methanol (HPLC grade, J.T. Baker, Phillipsburg, NJ, USA) followed by 10 ml of deionized water, except columns were not placed under vacuum. Terpenes were adsorbed onto the SPE column by adding 30 μ l of a 100 ng/ μ l standard solution and 3 ml of rumen fluid, plasma, or serum. After columns emptied by gravity flow, impurities were washed twice with 5 ml of deionized water until drips ceased. Terpenes were eluted with 1 ml of ethyl acetate (HPLC grade, Aldrich, USA) into 2 ml autosampler vials (slowly pushed through with 30 ml disposable syringe) and capped. Because the step in which columns were dried under vacuum (Kimball et al., 2004) was eliminated, eluate was refrigerated for a few hours and transferred to a freezer (–20 °C) overnight, and then the ethyl acetate phase was decanted into a 2 ml vial (to remove residual water) and stored at –20 °C prior to GC analysis.

2.4. Instrumentation and analytical conditions

Recoveries were measured by gas chromatography/flame ionization detector (Hewlett Packard GC 5890 Series II). Identity of impurities in test compounds and blanks was examined with a Finnigan Ion Trap MS (EI, 70 eV) coupled to a Varian (model 3400) GC. A DB-5 fused silica capillary column (30 m length, 0.25 mm i.d., 0.25 μ m film thickness, 5 mol/100 mol phenyl-methylpolysiloxane coating; J&W Scientific) was used in both instruments. Column conditions followed Adams (1995): injector temperature: 220 °C, transfer line temperature: 240 °C, detector temperature: 260 °C, initial column temperature: 60 °C: final column temperature 240 °C, ramp: 3 °C/min, and carrier gas: helium (flow rate: 1 ml/min) for both instruments. Split flow was 1 ml/min (GC/FID) and 20 ml/min (GC/MS). Injection size was 5 μ l (GC/FID) and 1 μ l (GC/MS). Other GC/FID parameters were: make up gas (He; 29 ml/min), detector air flow (300 ml/min), and detector H flow (30 ml/min).

2.5. Standards, blanks, and calculations

Standards (1, 2, 3, 4, and 5 ng/ μ l) for each terpene were injected (GC/FID, 1–5 ng/ μ l external standard curves, 5 μ l injection, 5 ml/min split) to determine linearity. Linearity for all standard curves ranged from R^2 of 0.9996 to 0.9982. Consequently, concentrations in eluate were estimated from peak area ratios using single point (3 ng/ μ l) external standards (mean of triplicate injections analyzed same day as unknowns but injected directly into GC). Recovery was calculated by dividing eluate terpene concentrations by 3 ng/ μ l (theoretical concentration assuming complete recovery; *i.e.*, all 3000 ng placed on column recovered in 1 ml of ethyl acetate). Terpene recovery from serum, plasma, and rumen fluid was examined for eight replicates per group with each fluid. Thus, 23 compounds were examined once per fluid, while two compounds were tested four times per fluid ($n = 32$).

Duplicate blanks for each fluid were run through the extraction procedure as described above except for terpene addition. Blanks were examined to verify no impurities were present in serum, plasma, or rumen fluid matrices with RT that interfered with peak integration or recovery calculation. Plasma blanks contained no impurity peaks that interfered with integration or calculations, while serum collected at the same time contained two peaks that interfered with a target terpene. A large peak (RT = 7.93 min) interfered with limonene integration, and a small peak (RT = 7.78 min) interfered with *p*-cymene. Rumen fluid stored at –80 °C was reasonably free of interfering peaks, with only one peak (RT = 9.79 min) that interfered with terpinolene. These three compounds were corrected by subtracting out the blank peak area prior to recovery calculations.

2.6. Statistical analysis

Terpene recovery was analyzed with the MIXED procedure (SAS, 2004). Effect of fluid on recovery of each compound was examined, with fluid as the independent factor tested with the residual as the error term. A separate analysis was conducted for 2-carene and longifolene to assess method consistency over time and influence of grouping on recovery. In that analysis, group was the independent factor and was tested with the residual as the error term. In the event of a significant *F* test ($\alpha = 0.05$), mean separation was conducted by least significant difference ($P < 0.05$).

3. Results

Recovery of each terpene was compared across the three fluids (Table 1). Two terpenes (2-carene and longifolene) were compared among groups within fluid as well (Table 2). Mean recoveries of 14 terpenes from serum (camphene, β -pinene, α -terpinene, *p*-cymene, *cis*- β -ocimene, 1,8-cineole, γ -terpinene, terpinolene, linalool, camphor, longifolene, β -caryophyllene, α -humulene, and caryophyllene oxide) were near 1 ng/ng. Four others (*cis*-sabinene hydrate, borneol, terpin-4-ol, and α -terpineol) had recoveries from serum near 1.2 ng/ng, and the remaining seven compounds (tricyclene, α -pinene, sabinene,

Table 1

Terpene recovery (ng/ng) from serum, plasma, and rumen fluid of sheep.

Chemical	Serum	Plasma	Rumen fluid	SEM	P value
Tricyclene	0.93a	0.92a	0.43b	0.033	0.0001
α -Pinene	0.88a	0.99b	0.54c	0.035	0.0001
Camphene	1.01a	1.08a	0.54b	0.040	0.0001
Sabinene	0.85a	0.85a	0.50b	0.023	0.0001
β -Pinene	0.96a	1.05b	0.55c	0.029	0.0001
Myrcene	0.91a	0.90a	0.52b	0.030	0.0001
2-Carene	0.80a	0.85b	0.48c	0.015	0.0001
3-Carene	0.86a	0.86a	0.42b	0.024	0.0001
α -Terpinene	0.98a	0.83b	0.40c	0.040	0.0001
<i>p</i> -Cymene ^a	1.00	1.08	0.92	0.049	0.0913
Limonene ^a	0.91a	0.94a	0.51b	0.032	0.0001
1,8-Cineole	1.02a	1.19b	1.05a	0.028	0.0004
<i>cis</i> - β -Ocimene	1.00a	0.89b	0.61c	0.030	0.0001
γ -Terpinene	1.01a	0.92a	0.56b	0.033	0.0001
<i>cis</i> -Sabinene hydrate	1.19a	1.01b	1.07b	0.034	0.0042
Terpinolene ^b	1.00	0.98	0.96	0.035	0.6766
Linalool	0.99a	1.01a	1.19b	0.028	0.0001
Camphor	1.00a	1.19b	1.15b	0.027	0.0001
Borneol	1.20a	1.04b	1.06b	0.028	0.0009
Terpin-4-ol	1.22a	1.12a,b	1.08b	0.031	0.0183
α -Terpineol	1.18a	1.07a,b	1.01b	0.041	0.0227
Longifolene	0.97a	1.01a	0.59b	0.014	0.0001
β -Caryophyllene	1.00a	1.07b	0.74c	0.021	0.0001
α -Humulene	1.00a	1.10b	0.56c	0.027	0.0001
Caryophyllene oxide	1.02a	1.20b	0.99a	0.028	0.0001

Means in a row followed by different letters (a, b, c) differ ($P < 0.05$); $n = 8$ for all but 2-carene and longifolene ($n = 32$).^a Recovery from serum corrected for impurity in blank at same RT by peak area subtraction.^b Recovery from rumen fluid corrected for impurity in blank at same RT by peak area subtraction.

myrcene, 2-carene, 3-carene, and limonene) were in the 0.8–0.95 ng/ng recovery range. Terpene recovery from plasma was also generally good (near unity), and again 2-carene recovery was lowest. Only six mean recoveries from plasma were below 0.9 ng/ng (sabinene, myrcene, 2-carene, 3-carene, α -terpinene, and *cis*- β -ocimene), and four others (1,8-cineole, camphor, terpin-4-ol, and caryophyllene oxide) were above 1.1 ng/ng. Serum and plasma recoveries differed ($P < 0.05$) for nearly half of the compounds (Table 1), but in most cases differed by less than 10%.

In contrast, terpene recoveries from rumen fluid were generally lower than for serum or plasma. Mean recovery from rumen fluid was near unity for eight terpenes (*p*-cymene, 1,8-cineole, *cis*-sabinene hydrate, terpinolene, borneol, terpin-4-ol, α -terpineol, and caryophyllene oxide), and above 1.1 ng/ng for two others (linalool and camphor). Eleven recovery values from rumen fluid were below 0.75 ng/ng (α -pinene, camphene, sabinene, β -pinene, myrcene, *cis*- β -ocimene, limonene, γ -terpinene, longifolene, β -caryophyllene, and α -humulene), and four others were below 0.5 ng/ng (tricyclene, 2-carene, 3-carene, and α -terpinene). Recovery from rumen fluid differed ($P < 0.05$) from the other two fluids for 16 compounds (Table 1), and was not different than either fluid in only two cases (*p*-cymene and terpinolene). Recovery of two terpenes (*p*-cymene and terpinolene) did not differ among fluids (main effect; $P > 0.05$).

Recovery of longifolene (Table 2) was consistent for serum and plasma among each of the four groups in which it was measured, but differed for rumen fluid, although groups 1 and 4 (first and last groups analyzed) were not different. In contrast, 2-carene differed by group for all fluids, but no obvious decline with time was observed. Recovery from group 1 was greater than other groups for plasma, and was greater than groups 2 and 3 for rumen fluid ($P < 0.05$).

Table 2

Recovery (ng/ng) of 2-carene and longifolene from serum, plasma, and rumen fluid of sheep.

Chemical ^a	Group 1	Group 2	Group 3	Group 4	SEM	P value
2-Carene, serum	0.73a	0.81a	0.74a	0.92b	0.036	0.0030
Longifolene, serum	0.95	0.97	0.98	1.00	0.029	0.7447
2-Carene, plasma	0.95a	0.81b	0.82b	0.83b	0.028	0.0042
Longifolene, plasma	1.04	0.99	1.02	0.99	0.034	0.6758
2-Carene, rumen fluid	0.60a	0.44b,c	0.40c	0.48b	0.021	0.0001
Longifolene, rumen fluid	0.66a	0.53b	0.57b	0.62a	0.016	0.0001

Means in a row followed by different letters (a, b, c) differ ($P < 0.05$); $n = 8$.

^a 2-carene and longifolene were present in each group. Groups also contained α -pinene, camphene, β -pinene, *p*-cymene, terpinolene, borneol, and β -caryophyllene (Group 1) sabinene, myrcene, limonene, camphor, and α -humulene (Group 2) tricyclene, 3-carene, 1,8-cineole, γ -terpinene, linalool, terpin-4-ol, and caryophyllene oxide (Group 3) and α -terpinene, *cis*- β -ocimene, *cis*-sabinene hydrate, and α -terpineol (Group 4).

4. Discussion

Some general consistencies were noted within and among fluid matrices and chemical subclasses. Oxygenated compounds were typically recovered to a greater extent and hydrocarbon compounds (particularly monoterpenes) were less efficiently recovered with this method (likely a function of the SPE adsorbent properties). The four compounds recovered from serum in greatest amounts (~1.2 ng/ng) were all oxygenated monoterpenes containing a hydroxyl group, and the four with highest recovery from plasma were three oxygenated monoterpenes and one oxygenated sesquiterpene; however, only terpin-4-ol was present in both groups. The seven compounds with lowest recovery from serum were all hydrocarbon monoterpenes, and the six lowest recoveries from plasma were also all hydrocarbon monoterpenes, but only four were contained by both groups. Although recovery was generally lowest from rumen fluid, the pattern was similar, in that two oxygenated monoterpenes had recoveries near 1.2 ng/ng, while eight hydrocarbon monoterpenes and three hydrocarbon sesquiterpenes were recovered at <0.75 ng/ng and four other hydrocarbon monoterpenes were recovered at <0.5 ng/ng.

Patterns that deviated from these general tendencies were occasionally observed. For example, 1,8-cineole exhibited similar recovery from serum and rumen fluid (near unity), while recovery from plasma was approximately 20% greater ($P < 0.05$). Conversely, recoveries of *cis*-sabinene hydrate and borneol from plasma and rumen fluid were similar but serum recovery was approximately 20% greater. This variable recovery of these three oxygenated monoterpenes from the different matrices also suggests that recovery estimates from serum and plasma are not interchangeable. Even highly structurally similar compounds occasionally exhibited dissimilar recovery patterns. For example, sabinene and β -pinene are both bicyclic alkenes with one exocyclic double bond that reach the detector within five seconds of each other during chromatographical analysis and have almost identical fragmentation patterns. Yet, recovery of sabinene was substantially lower than β -pinene in all fluids (Table 1). Thus, caution should be exercised when making the assumption that compounds of similar size and structure will make suitable surrogate standards or substitute for another analyte using this procedure.

Recoveries of *p*-cymene, 1,8-cineole, and camphor from plasma in this study are somewhat higher than recoveries of those three compounds observed by Kimball et al. (2004). To our knowledge, no data are available for comparison that used a similar procedure with other fluids (serum or rumen fluid) or terpenes other than the three mentioned previously. Although it is conceivable these differences are due to modifications made to the procedure described by Kimball et al. (2004), the modifications were minor. First a different SPE column was utilized, but both were C_{18} -500 mg columns. Second, the use of vacuum to facilitate column flow was eliminated because it improved recovery during preliminary tests conducted to maximize extraction efficiency at biologically relevant concentrations. Consequently, a step was added in which eluate was frozen in order to decant residual water. A few other procedural aspects were incorporated in response to issues encountered specifically with rumen fluid that are not true modifications since Kimball et al. (2004) examined only plasma. Rumen fluid appeared to decay during storage (particularly with repeated freezing/thawing), contributing to impurities that interfered with peaks of interest. Both high-speed centrifugation and re-centrifuging rumen fluid immediately before use improved recoveries and column flow, and were therefore included in the procedure. Storage of rumen fluid at -80°C appeared to minimize this problem and was also incorporated into the final procedure. Impurities were also inconsistent among blank fluids collected in different batches and different time of day (particularly rumen fluid) during preliminary tests. Interference peaks in blanks likely vary with animal species, diet, time of collection relative to feeding, and a host of other experimental conditions, and should be examined in each experiment. While not addressed specifically in this study, rumen fluid should be frozen quickly to accurately reflect actual sampling time, because terpene disappearance from rumen fluid is quite rapid in some cases and occurs at different rates for different compounds (Broudiscou et al., 2007; Malecky et al., 2009).

It is unclear why recoveries in excess of 1 ng/ng were observed in a few instances. Obviously, small deviations can be explained by experimental error. Structural alteration or interconversion among compounds within a group, while unlikely, might contribute to overestimation of recovery. Recovery estimates for the two compounds present in each group were not always consistent, but no clear patterns emerged. Since compounds are added immediately before extraction, this effect is likely unrelated to sample stability, although fluid storage time (matrix decay discussed earlier) may be a factor. Although unlikely, each group also contains a different array of compounds that could potentially impact their recovery. However, this observation points to the need to assess recovery at the same time unknowns are analyzed.

In conclusion, this modified procedure can be used to measure an array of mono- and sesquiterpenes from blood and rumen fluid. However, differences in recovery based on both structure and sample matrix indicate a need to correct terpenes for recovery of that specific compound to determine absolute amount. Differential recovery of compounds indicates that caution should be exercised in generalizing responses of compounds with similar structures. Ideally, each analyte in unknown samples should be corrected for recovery of the same compound in the same matrix at the same time for each set of experimental conditions.

Acknowledgements

The authors are grateful for the assistance of D.M. Hallford with blood collection and Y.F. Wang in laboratory analyses.

References

- Adams, R.P., 1995. Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry. Allured Publishing, Carol Stream, IL, USA.
- Broudiscou, L.P., Cornu, A., Rouzeau, A., 2007. *In vitro* degradation of 10 mono- and sesquiterpenes of plant origin by caprine rumen micro-organisms. J. Sci. Food Agric. 87, 1653–1658.
- Cardoza, P.W., Calsamiglia, S., Ferret, A., Kamel, C., 2004. Effects of natural plant extracts on ruminal protein degradation and fermentation profiles in continuous culture. J. Anim. Sci. 82, 3230–3236.
- Chizolla, R., Hochsteiner, W., Hajek, S., 2004. GC analysis of essential oils in the rumen fluid after incubation of *Thuja orientalis* twigs in the Rusitec system. Res. Vet. Sci. 76, 77–82.
- Kimball, B.A., Dziba, L.E., Johnston, J.J., Provenza, F.D., 2004. Chromatographic analysis of sagebrush monoterpenes in plasma. J. Chromatogr. Sci. 42, 245–249.
- Malecky, M., Broudiscou, L.P., 2009. Disappearance of nine monoterpenes exposed in vitro to the rumen microflora of dairy goats: effects of inoculum source, redox potential, and vancomycin. J. Anim. Sci. 87, 1366–1373.
- Malecky, M., Broudiscou, L.P., Schmidely, P., 2009. Effects of two levels of monoterpene blend on rumen fermentation, terpene and nutrient flows in the duodenum and milk production in dairy goats. Anim. Feed Sci. Technol. 154, 24–35.
- Newbold, C.J., McIntosh, F.M., Williams, P., Losa, R., Wallace, R.J., 2004. Effects of a specific blend of essential oil compounds on rumen fermentation. Anim. Feed Sci. Technol. 114, 105–112.
- SAS, 2004. SAS Documentation. Version 9.1.3. SAS Institute Inc., Cary, NC, USA.